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Unusual small plasmids carrying the novel resistance genes *dfrK* or *apmA* isolated from methicillin-resistant or -susceptible staphylococci

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Objectives: The aims of this study were to identify small staphylococcal plasmids that carry either the trimethoprim resistance gene *dfrK* or the apramycin resistance gene *apmA* and analyse them for their structure and organization with regard to their potential role as precursors of large multiresistance plasmids that carry these genes.

Methods: Trimethoprim- or apramycin-resistant staphylococci from the strain collections of the two participating institutions were investigated for the presence of plasmid-borne *dfrK* or *apmA* genes. The *dfrK*- or *apmA*-carrying plasmids were sequenced completely and compared with sequences deposited in the databases.

Results: Two small plasmids, the 4957 bp *dfrK*-carrying plasmid pKKS966 from porcine *Staphylococcus hyicus* subsp. *hyicus* and the 4809 bp *apmA*-carrying plasmid pKKS49 from porcine methicillin-resistant *Staphylococcus aureus* were identified. Structural analysis revealed that both plasmids had a similar organization, comprising a single resistance gene (*dfrK* or *apmA*), a plasmid replication gene (*rep*) and three partly overlapping genes for mobilization proteins (*mobA*, *mobB* and *mobC*). Comparisons showed 71%–82% amino acid identity between the Rep and Mob proteins of these two plasmids; however, distinctly lesser percentages of identity to Rep and Mob proteins of staphylococci and other bacteria deposited in the databases were detected.

Conclusions: Both plasmids, pKKS966 and pKKS49, appeared not to be typical staphylococcal plasmids. The homology to larger plasmids that harbour the genes *apmA* and/or *dfrK* was limited to these resistance genes and their immediate upstream and downstream regions and thus suggested that these small plasmids were not integrated into larger plasmids.

Keywords: MRSA, trimethoprim resistance, apramycin resistance, horizontal gene transfer

Introduction

During recent years, several novel resistance genes, including the trimethoprim resistance gene *dfrK* and the apramycin resistance gene *apmA*, have been identified among methicillin-resistant *Staphylococcus aureus* (MRSA) of clonal complex (CC) 398 from animals.[1–3] As most of these novel resistance genes have been identified in MRSA isolates from pigs,[3] it appears as if there could be a potential porcine reservoir of antimicrobial resistance genes. Both genes, *dfrK* and *apmA*, were usually co-located with other resistance genes on multiresistance plasmids of ~40 kb.[1–3] The *dfrK* gene was commonly linked to the tetracycline resistance gene *tet*(L), as confirmed for plasmids in MRSA of sequence type (ST) 125 of human origin[4] or MRSA CC398 from pigs,[5] dairy cattle[6] or food of poultry origin.[7] Some of these *tet*(L)-*dfrK*-carrying plasmids also carried additional resistance genes, such as *cfr* and *aadD* [also known as *ant(4′)-Ia*][4] or *aadD* and *vga*(C).[3] In addition, *dfrK* [without *tet*(L)] has been identified as part of transposon Tn*559*, which was first detected in a porcine methicillin-susceptible *S. aureus* CC398.[8] The origin of both genes, *dfrK* and *apmA*, is unknown and analysis of their genetic environment has not revealed their source and how they became incorporated in the aforementioned multiresistance plasmids.

The aims of this study were to identify small staphylococcal plasmids that carry the trimethoprim resistance gene *dfrK* or the apramycin resistance gene *apmA* as the sole resistance gene. Analysis of such plasmids for their structure and organization might provide insight into whether these small plasmids could have acted as precursors of large multiresistance plasmids that carry *apmA* and/or *dfrK* in addition to other resistance genes.

Materials and methods

To find out whether there are plasmids that carry only *dfrK* or *apmA*, trimethoprim- or apramycin-resistant staphylococcal isolates from the strain collections of both participating institutions were screened for the presence of the genes *dfrK* and *apmA* by previously described PCR assays.[2,5] Plasmids were prepared and transferred into *S. aureus* RN4220 via electrotransformation. Transformants were selected on trimethoprim-containing (30 mg/L) or apramycin-containing (20 mg/L) medium. The plasmids were digested with suitable enzymes, cloned into pBluescript II SK+ and sequenced completely. Two small plasmids, one harbouring *dfrK* and the other harbouring *apmA*, were identified. Their sequences have been deposited in the EMBL database under accession numbers FN677368 (pKKS966) and HE611647 (pKKS49).

Results and discussion

The dfrK-carrying trimethoprim resistance plasmid pKKS966

The *dfrK*-carrying plasmid pKKS966 was isolated from a *Staphylococcus hyicus* subsp. *hyicus* isolate obtained from a sow with a skin infection during the BfT-GermVet study conducted between 2004 and 2006 in Germany. The isolate showed resistance to trimethoprim (MIC ≥512 mg/L) and enrofloxacin (MIC 4 mg/L). Plasmid pKKS966 had a size of 4957 bp and a GC content of 36.1%. Sequence analysis identified five open reading frames (Figure 1). The first reading frame coded for a potential plasmid replication protein of 219 amino acids. The Rep protein was next related to Rep proteins of small plasmids isolated from *Selenomonas ruminantium* (201 amino acids, 48% identity, accession number NP_862700) or from *Bacillus mycoides* (186 amino acids, 41%, accession number NP_981975.1). The fifth reading frame was the *dfrK* gene, which encoded a 163 amino acid dihydrofolate reductase. This *dfrK* gene was identical to previously identified, plasmid-located *dfrK* genes from MRSA ST398 and ST125.[1–3] The encoded protein DfrK differed in six amino acids from a DfrK of an equine *S. aureus* isolate (accession number CBL80435.1) and in seven amino acids from the DfrK located on Tn*559* from a methicillin-susceptible *S. aureus* ST398 isolate.[8] The *dfrK*-flanking regions in pKKS966 showed homology to plasmid pKKS2187[1] for 166 bp upstream of *dfrK* and for 377 bp downstream of *dfrK*. To the best of our

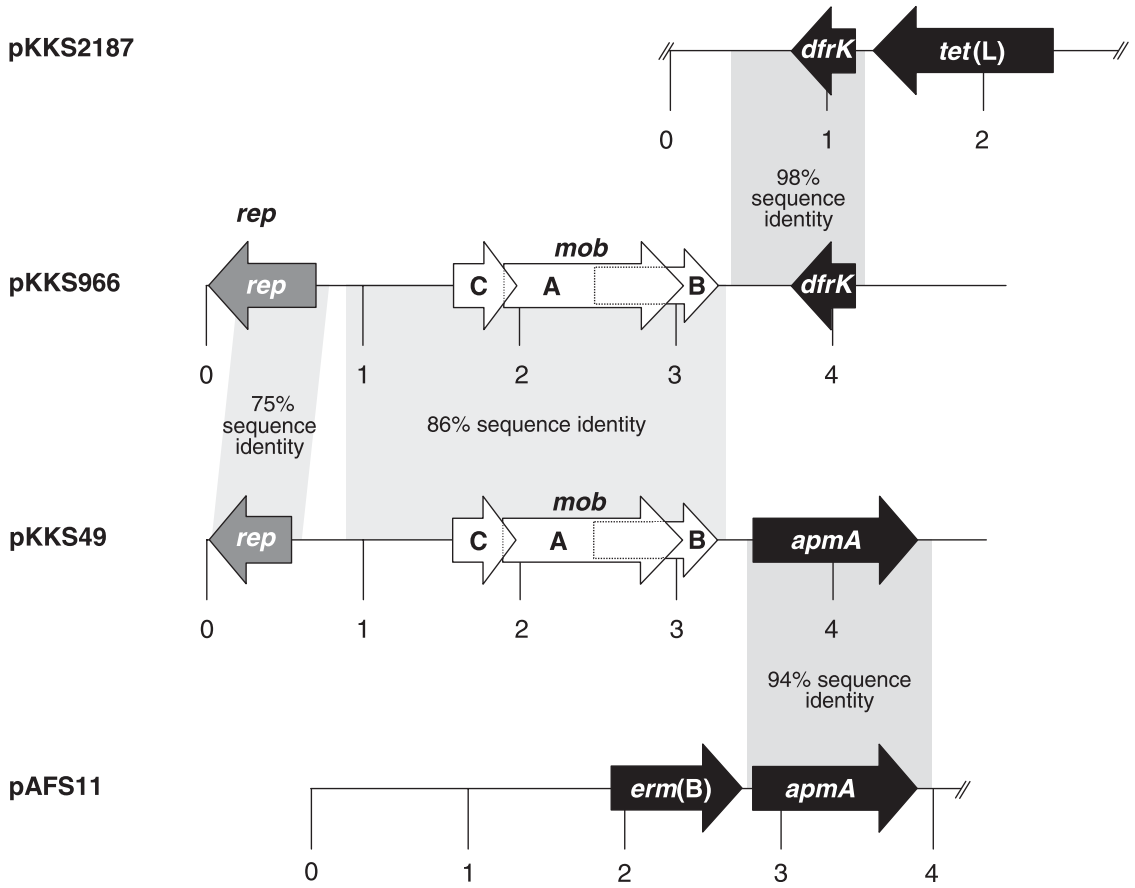

Figure 1. Maps of the plasmids pKKS966 (FN677368) and pKKS49 (HE611647) in comparison with parts of the *dfrK*-carrying plasmid pKKS2187 (FM207105)[1] and the *apmA*-carrying plasmid pAFS11 (FN806789).[2] The arrows indicate the extents and directions of transcription of the genes *dfrK* (trimethoprim resistance), *tet*(L) (tetracycline resistance), *rep* (plasmid replication), *mobA*, *mob* and *mobC* (plasmid mobilization), *apmA* (apramycin resistance) and *erm*(B) (macrolide/lincosamide/streptogramin B resistance). Resistance genes are coloured in black, *mob* genes in white and *rep* genes in grey. The region of homology between the plasmids is marked by grey shading. A distance scale in kb is given below each map. The maps of plasmids pKKS966 and pKKS2187 have been redrawn from the corresponding database entries to match the reading frames in pKKS49.

knowledge, this is the first description of the *dfrK* gene in a staphylococcal species other than *S. aureus*. The three remaining reading frames of pKKS966 coded for Mob proteins. The *mobA* gene encoded a putative relaxase of 382 amino acids, with the best matches to a protein from a whole genome sequence of *Staphylococcus hominis* (321 amino acids, 56%; accession number ZP_04060076.1), but also to proteins encoded by plasmids from *Staphylococcus epidermidis* (337 amino acids, 51%; accession number AAD02405.1) or *S. aureus* (336 amino acids, 51%; accession number EHT35286.1). The *mobC* gene encoded a protein of 105 amino acids, which is most closely related to a protein from *Lactobacillus malefermentans* (123 amino acids, 52%; accession number ZP_09441588.1). In contrast, the MobB protein of 182 amino acids exhibited only 29% amino acid identity to a MobB protein of *Pediococcus pentosaceus* (189 amino acids; accession number AAD25895).

The apmA-carrying apramycin resistance plasmid pKKS49

The *apmA*-carrying plasmid pKKS49 originated from an MRSA ST398 isolate obtained from a dust sample taken in a holding with breeding pigs in Portugal as part of the EU baseline study. The isolate was resistant to apramycin (MIC ≥32 mg/L), tetracycline (MIC 64 mg/L), clindamycin (MIC ≥128 mg/L), tiamulin (MIC ≥128 mg/L) and oxacillin (MIC 16 mg/L). In addition to pKKS49, it also harboured the small *vga*(C)-carrying plasmid pCPS49.[9] Plasmid pKKS49 had a size of 4809 bp and a GC content of 38.6%. Five reading frames were identified (Figure 1). The first reading frame coded for a potential plasmid replication protein of 216 amino acids. This Rep protein was distantly related to known Rep proteins, with the best match of 71% amino acid identity to the Rep protein of plasmid pKKS966. Three partly overlapping reading frames for a 382 amino acid MobA protein, a 182 amino acid MobB protein and a 109 amino acid MobC protein were detected. The MobA, MobB and MobC proteins showed best matches of 82%, 75% and 77% amino acid identity to the respective proteins encoded by the *mob* genes of plasmid pKKS966. The sequence identities to pKKS966 of these two gene regions are shown in Figure 1. The fifth reading frame encoded a 274 amino acid protein that differed by 12 amino acids from the same-sized ApmA protein of plasmid pAFS11 from a bovine MRSA ST398.[2] Analysis of the sequences flanking this *apmA* gene variant identified homology to plasmid pAFS11 for only 72 bp in the *apmA* upstream and 64 bp in the *apmA* downstream region. While *dfrK* has been described recently in isolates from Spain,[3,10] this is the first identification of the novel gene *apmA* in an isolate not originating from Germany.

Relevance and origin of small plasmids

The results of this study confirmed that the novel resistance genes *dfrK* and *apmA* occur not only on large multiresistance plasmids, but also on small resistance plasmids that do not carry additional resistance genes. These plasmids showed an overall organization that is similar to that of small staphylococcal resistance plasmids.[7] However, a closer look at the similarities at the amino acid level revealed that the pKKS966- and pKKS49-associated Rep and Mob proteins are only distantly related to corresponding proteins of staphylococci. In addition, the observation that the GC contents of 36.1% (pKKS966) or 38.6% (pKKS49) are higher than the GC content of sequenced staphylococcal genomes [*S. epidermidis* RP62A (32.1%), *Staphylococcus haemolyticus* JCSC1435 (32.8%), *S. aureus* N315 (32.8%), *Staphylococcus saprophyticus* ATCC15305 (33.2%) and *Staphylococcus carnosus* TM300 (34.6%)] might support the assumption that these two plasmids do not originate from staphylococci.[11] Comparative analysis of the *dfrK*-flanking regions in plasmids pKKS966 and pKKS2187 and the *apmA*-flanking regions in plasmids pKKS49 and pAFS11, respectively, did not identify specific sequences or structures that might point towards recombination processes between the small plasmids and the corresponding larger *dfrK*- or *apmA*-carrying plasmids. Consequently, it is questionable whether these two small plasmids acted as sources for the incorporation of *dfrK* or *apmA* into larger plasmids.

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Transparency declarations

None to declare.

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